

5 **A RAPID IMMUNOASSAY OF ANTHRAX PROTECTIVE ANTIGEN IN
VACCINE CULTURES AND BODILY FLUIDS BY FLUORESCENCE
POLARIZATION**

CROSS REFERENCE TO RELATED APPLICATION

10 This application claims priority to U.S. Provisional application 60/457,940 filed
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FIELD OF THE INVENTION

The inventive subject matter relates to a competitive fluorescence method for
estimating the concentration in a sample of *Bacillus anthracis* protein or specific
15 antibody. The method contemplates the use of FLT, FRET or FP.

BACKGROUND OF INVENTION

Oral fluids have been increasingly recognized as acceptable alternatives to serum
for use in diagnostic tests for certain hormones, drugs, antibodies and antigens. However,
20 diagnostic assays utilizing saliva and other oral fluids appear as entries in the National
Library of Medicine MEDLARS database with a frequency of only 1 in 30 and 1 in 100,
respectively, when compared to entries in which blood serum is reported. Oral fluids are
collected without pain, needle sticks, or religious and social prohibitions, and use of these
involves minimal risk or exempt protocols for the use of human subjects. The Department
25 of Defense (DoD) has made salivary diagnostics a Future Naval Capability (FNC), and
both the Office of Naval Research (ONR) and the Military Infectious Disease Research
Program (MIDRP) of the US Army have funded projects to investigate the use of oral

5 fluids or saliva to diagnose disease or monitor immunization status. The military has had
a long-standing interest in saliva with ONR compiling in 1960 a review of all saliva
references in the literature from 1888-1957 (1). In 1965, the US Army Medical Research
Development Command supported a contract that published one of the first reports of
human salivary antibodies to indigenous bacteria (2). The United States Department of
10 Agriculture (USDA) recently sponsored a symposium to advance non- or minimally-
invasive technologies to monitor health and nutritional status in the Special Supplemental
Nutrition Program for Women, Infants and Children (WIC) using saliva as a diagnostic
tool (3, 4). These considerations and continuing improvements in standardization of
collection methods make these body fluids the diagnostic media of choice in the 21st
15 century.

Recent reviews of the clinical chemistry and microbiology of saliva (5), the roles
of saliva in health and disease (6), in diagnosing periodontal disease (7), and as a
diagnostic fluid (8) indicate the growing prominence this matrix plays in medical
diagnostics (9,10). Saliva (6) and oral fluid (11) are distinct biochemically. They
20 generally reflect the serum pool, but neither is a passive ultrafiltrate (passive requires no
direct ATP use) of serum as previously thought (12). The presence of mucins, the
polyanionic glycoproteins that increase salivary viscosity, and oral flora have been
largely responsible for the lack of popularity of oral fluids in clinical research. Saliva
presents several challenges for diagnostics: limited reference values have been published
25 and the standardization of sample collection has fallen behind serum (13). Standardized
saliva (14) and oral fluid (15) collection devices have only recently become available and
should contribute to further investigations. Antibody containing oral fluids, gingival

5 crevicular fluid (GCF) and oral mucosal transudate (OMT) arise due to hydrostatic pressure of the capillaries and venules associated with the lingual or buccal epithelium. They offer less variation than saliva and the best alternative to serum for antibody detection (16). GCF is similar to serum in protein composition but is significantly lower in protein concentration, being about 3% of the protein levels in blood (17,18). GCF
10 volume is about one percent of total saliva volume in the healthy mouth (19). GCF is obtained by inserting an absorbent paper into the pocket or sulcus of a tooth (between the tooth and gingiva) after clearing the supragingival plaque (20). Its medical and dental use has not been reviewed since the 1970's (21,22). OMT is 3-4 fold higher in protein concentration than saliva based on the IgG obtained using this device and is collected by
15 placing a thick pad against the buccal mucosal surface juxtaposed between the parotid duct and the gingival crest (11). A current OMT device uses a salt-impregnated pad that is subsequently treated to release the antibody-containing fluid and retain the glycoproteins on the pad yielding "oral fluid". One such oral fluid collection device has been licensed by Abbott Laboratories and eliminates the necessity for venipuncture (23).

20 The oral cavity as an immunological entity has been reviewed with respect to oral diseases (24) and microbiology (19). The use of oral fluids in diagnostic immunology includes detection of HIV (25); measles, mumps and rubella (26); hepatitis A (27); B (28); and C (29); *Helicobacter pylori* (30); dengue (31); and Chagas' disease (32). The current review will focus on the use of fluorescence polarization (FP)-based tests to
25 detect antibodies to anthrax vaccine and to tuberculosis exposure using saliva, GCF or OMT as a test fluid.

5 Currently excepted methods for the clinical evaluation of patients using serum
and oral fluids include enzyme-linked immunosorbent assays (ELISA), agglutination or
radioimmunoassays (RIA). However, FP has distinct advantages over previous methods.
Because FP is conducted in a fluid environment and because polarization is a general
property of fluorescent molecules, FP assays have the potential to be less susceptible to
10 non-specific interactions occurring at the cell surface and to interferences present in non-
homogeneous sample fluids. However, despite the clear advantages of FP in diagnostics,
only a limited number of non-commercial laboratories have adopted FP for detection of
antibody in serum samples (33-35). High throughput screening (HTS) laboratories, on
the other hand prefer FP based assays as a component of an automatable system capable
15 of real-time evaluation of samples that are robust, flexible, sensitive and consistent with
historical data (36).

Fluid-based assays are highly adaptable to the direct evaluation of oral fluids,
saliva and serum for exposure to infectious agents including HIV, measles, mumps,
rubella, hepatitis, *Helicobacter pylori*, dengue disease (37-43). Salivary and oral fluid
20 assays have been increasingly recognized as a better, non-invasive alternative to serum-
based diagnostics in detecting certain hormone, drug, antibody and antigen detection (44,
45).

Infectious disease rates and immunization strategies are critical to public health as
well as a component of military preparedness. An integral component of the U.S.
25 immunization strategy is the production of effective vaccines against agents such as
Bacillus anthracis (anthrax) as well as other emerging organisms such *Mycobacterium*
tuberculosis and *M. bovis*, the causative agents of tuberculosis (47).

5 Pathology due to *B. anthracis* infection is primarily due to the release by the
organism of “protective antigen” (PA) in association with lethal factor (LF) and edema
factor (EF) (48). The complete DNA and protein sequence of PA has been published and
its three-dimensional structure is known from x-ray crystallography (49). The
characteristics and biological functions of the four domains of PA are also available
10 permitting selection of epitopes within the domains based on antigenic properties (49-53).
In animal studies, as well as studies of natural human infection, it was shown that
individuals who survived an infection produced antibodies to PA suggesting its
importance in protection (54).

Vaccination for anthrax occurs for all Department of Defense personnel deploying
15 to at-risk areas of operation. Furthermore, research personnel working with infectious
strains of anthrax are vaccinated, prophylactically. The current human anthrax vaccine
(adsorbed) (AVA) licensed in the United States contains aluminum hydroxide adjuvant
and consists mostly of PA from an attenuated, non-encapsulated strain of *B. anthracis*
(55,56). Several recombinant PA experimental plasmids have been produced which
20 could be important as a vaccine component against the disease.

The production of bacteria or their products for use in vaccines requires routine
monitoring of bacterial growth to obtain the material in optimum quantities and in a form
most suitable for eliciting immunity. Expression of specific proteins, depending on the
organism, is to a great extent dependent on the phase of bacterial cell growth in culture
25 and the density of the protein or organism. Routine monitoring of growing cultures is,
therefore, important. Assays for bacterial growth monitoring, must be relatively accurate,
sensitive and able to be conducted in near real-time. Furthermore, the assay must be able

5 to operate in the face of turbid media containing interfering substances, not related to the target protein being measured.

Current methods available to monitor bacterial growth include optical density measurement, which gives an estimation of bacterial density but no direct measure of specific protein concentrations being produced. More accurate methods include Western blot; enzyme-linked immunosorbent assay (ELISA); dot blots; which is a form of ELISA and polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE). ELISA methods, however, are inadequate due to either low sensitivity or erroneous results caused by interference effects by media constituents. SDS-PAGE methods are inadequate because they do not give real time information such that a culture composition cannot be estimated with accuracy given the time span between culture SDS-PAGE and interpreting the SDS-PAGE results.

Fluorescent polarization (FP) technology is capable of rapid, real-time, sensitive evaluation of fluid phase antigens with high specificity (57, 58). FP based assays are predicated on the principle that polarized incident visible or ultraviolet light that illuminates a fluorochrome causes subsequent polarized fluorescence with emission at a longer wavelength. However, molecules in solution are capable of rotation. Therefore, polarized light striking a fluorescent molecule loses polarization due to rotation of the molecule. Solutions containing slower turning, large molecule-fluorochrome complexes tends to stay polarized longer verses situations where smaller labeled molecules are present. In order to accommodate molecules of different sizes (up to 10^7 kDa molecular weight), different fluorochromes can be selected (59).

5 Combining fluorochrome-labeled antigen or peptide with antibody results in an increase in FP, as measured in arbitrary millipolarization (mP) units. The smaller the fluorescent antigen, the greater the increase in mP units that is measured upon binding to its corresponding antibody, since mP depends upon the partial specific volume (approximate molecular weight in solution) of the labeled substance. The dependence is
10 non-linear but is describable in a Perrin equation (66).

FP antigen-antibody binding assays require only the mixing of fluorescent reagent (antigen) with the sample (containing antibody) in a liquid buffer. In a rapid diagnostic format, essentially two FP readings are necessary; a base-line reading and a reading after a specified time. The FP value increases as binding of antigen and antibody occurs in a
15 direct binding assay. The difference in FP between a fluorescent antigen of 10 kDa initially and the fluorescent complex consisting of it and IgG, for example, results in a measurable association using less than saturating antibody concentrations (60).

A distinct advantage of FP technology is that assays can accommodate somewhat cloudy solutions, such as bacterial suspensions or variations in total fluorescence that are
20 present in non-homogenous solutions, such as bacterial broth, urine or oral fluids. Furthermore, FP assays can be designed to accommodate significant variation in pH in fluid samples, such as in some media or in saliva or urine, by utilizing different pH-independent fluorochromes (61).

Currently, FP assays are in used to measure different types of binding reactions,
25 to follow proteolytic reactions and to measure various enzymatic or receptor binding reactions (62, 63). In clinical settings, FP assays are used to measure the level of drugs, hormones or antibiotics in blood plasma (64). ELISA, RIA and immunoprecipitation

5 assays are the most accepted methods for detection of antibody in serum samples (60, 65, 66). However, FP, because of its advantages over other detection methods is well-suited as a diagnostic tool for analyzing formerly difficult to evaluate samples such as oral fluids and saliva, in addition to serum, for the quantitative assessment of specific antibody, diagnostic markers, drugs, chemicals and infectious or biohazardous agents.

10 A further aspect of the invention is the detection and quantitation of protective antigen, lethal factor and edema factor from *Bacillus anthracis* in saliva, blood, oral fluids or other bodily fluids and tissues. Current vaccines to *B. anthracis* are directed against PA. Furthermore, because of the importance of lethal factor and edema factor in the pathology of anthrax, detection of these genes in body fluids or broth cultures is also
15 important. Therefore, an inventive aspect is the specific, sensitive and rapid detection of PA in bodily fluids including saliva, and oral fluids and environmental samples.

SUMMARY OF THE INVENTION

Current methods for the monitoring of growing broth cultures of bacteria for
20 vaccine production, including ELISA and SDS-PAGE, are inadequate. ELISA exhibits high levels of interference by media constituents leading to low fidelity of results and concomitant low sensitivity of detection. SDS-PAGE, like ELISA is extremely time consuming making the method unsuitable for routine monitoring. Therefore, a method that is sensitive, rapid and that gives predictable results is important in order to optimize
25 production of bacterial vaccine components, including the vaccine components for *B. anthracis*, protective antigen (PA).

5 An object of the invention is an assay for the rapid detection and quantitation of
bacteria in culture media or broth of growing cultures of *B. anthracis* by competitive
fluorescent polarization (FP), fluorescence lifetime (FLT) analysis or fluorescence
resonance energy transfer (FRET). Another object of the invention is a rapid, real-time
detection method for monitoring growing bacterial cultures or bodily fluids. A still
10 further object is an assay capable of sensitive, rapid detection of bacterial media or bodily
fluids without interference from sample constituents. A still further object is the real-
time quantitation of protective antigen (PA) in growing cultures of *B. anthracis*. A still
further object is the quantitation of lethal factor (LF) in growing cultures of *B. anthracis*.
still further object is the quantitation of edema factor (EF) in growing cultures of *B.*
15 *anthracis*. A still further object is the detection of PA, LF and EF in bodily fluids,
including saliva and oral fluids and in environmental samples.

These and other objects of the invention are accomplished by an assay employing
fluorescent polarization in a competitive assay procedure.

BRIEF DESCRIPTION OF THE DRAWINGS

20 Figure 1. Diagram showing expected change in polarization at various concentrations of
competitive protein. For complete description, see example 1.

DETAILED DESCRIPTION

Fluorescent polarization holds great promise as an assay method for frequent,
routine, cost-effective means of obtaining quantitation of bacterial proteins in
25 growing bacterial broth cultures. Fluorescent molecules emit polarized fluorescence
when they absorb polarized light at a specific wavelength. However, inherent in
molecules in solution is their tendency to rotate. When polarized light strikes the

5 molecules in solution, the emitted light does not remain polarized because the
molecule is rotating rapidly in solution. The rate of rotation, however, is dependent
on the size of the molecule. Therefore, an antigen/antibody complex will have an
inherently slower rate of rotation causing more of the polarized fluorescence to be
emitted in the same plane as the incident light. The polarization-based readouts are
10 less susceptible to environmental interferences, such as pH, cloudiness, and particles
in the assay compartment than other light-based assays. FP is fundamentally and
theoretically different from most color based photometric techniques and gains its
analytical advantage by this intrinsic property of being independent of concentration.
The current application utilizes FP technology in a competitive assay to quantitate
15 bacterial protein concentration in growing bacterial broth cultures. The invention is
designed to be capable of determining the concentration of antigen in fermentation
broth.

An aspect of the invention is a competitive method for estimating the
concentration of *Bacillus anthracis* proteins such as protective antigen (PA), lethal
20 factor (LF) and edema factor (EF). The assay method incorporates the broad
detection technologies of fluorescence polarization (FP), fluorescence life-time (FLT)
and fluorescence resonance energy transfer (FRET). The general scheme of the assay
includes the following steps:

- a. intermixing a sample, suspected of containing a *B. anthracis* PA, LF or EF
25 with a specific antibody and a competitive reagent, suitable as a ligand for
the specific antibody and labeled a fluorochrome;

- 5 b. incubating the competitive reagent and specific antibody for 15 seconds to
 5 minutes, depending on suspected concentration of the target antigen in
 the sample;
- c. detecting the binding interaction of PA, LF or EF and specific antibody.

 Detection of specific agent would then be by either a change in fluorescence
10 polarization or a change in fluorescence life-time, depending on whether the technology
incorporated was FP or FLT, respectively. If the method incorporated FRET, then
detection is by sensitized fluorescence of the acceptor or by quenching of donor
fluorescence or by fluorescence depolarization. Furthermore, the competitive reagent
used in the assay can be either native or recombinant PA, LF or EF or fragments thereof.

15 Furthermore, an aspect of the invention is that different fluorochromes can be utilized
in order to optimize results, including the incorporation of pH-independent
fluorochromes. The fluorochromes that are included as an aspect of the invention
include: 7-AAD, Acridine Orange, Alexa 488, Alexa 532, Alexa 546, Alexa 568, Alexa
594, Aminonaphthalene, Benzoxadiazole, BODIPY 493/504, BODIPY 505/515, BODIPY
20 576/589, BODIPY FL, BODIPY TMR, BODIPY TR, Carboxytetramethylrhodamine,
Cascade Blue, a Coumarin, Cy2, CY3, CY5, CY9, Dansyl Chloride, DAPI, Eosin,
Erythrosin, Ethidium Homodimer II, Ethidium Bromide, Fluorescamine, Fluorescein,
FTC, GFP (yellow shifted mutants T203Y, T203F, S65G/S72A); Hoechst 33242,
Hoechst 33258, IAEDANS, an Indopyras Dye, a Lanthanide Chelate, a Lanthanide
25 Cryptate, Lissamine Rhodamine, Lucifer Yellow, Maleimide, MANT, MQAE, NBD,
Oregon Green 488, Oregon Green 514, Oregon Green 500, Phycoerythrin, a Porphyrin,
Propidium Iodide, Pyrene, Pyrene Butyrate, Pyrene Maleimide, Pyridyloxazole,

5 Rhodamine 123, Rhodamine 6G, Rhodamine Green, SPQ, Texas Red, TMRM, TOTO-1, TRITC, YOYO-1, vitamin B12, flavin-adenine dinucleotide, and nicotinamide-adenine dinucleotide.

The assay method is generally adaptable to the detection of PA, LF or EF in bacterial culture media as well as in other bodily fluids including: saliva, oral rinse expectorant,
10 oral fluid including oral mucosal transudate and gingival crevicular fluid, urine, sweat, tears, blood, serum, stool, gastric fluid, synovial fluid, phlegm, and other clinical and laboratory specimens and samples. The time of assay will depend on expected concentration in the sample. For bacterial cultures that has progressed near or past the logarithmic phase of its growth curve, it is estimated that 30 minutes or less will be
15 required for incubation with specific antibody.

The results of test samples will be compared to results obtained from negative or positive control results, using either FP, FLT or FRET technology. Negative controls will contain the same fluorochrome-labeled competitive reagent as used in the test sample. A positive control can also contain the same fluorochrome-labeled competitive
20 reagent as in the test sample but will be exposed to a known amount of said protein. The measured results of the test samples will then be compared to either or both of the negative or positive controls. It is estimated that the sensitivity of detection of *B. anthracis* protein will occur down to at least 5 µg/ml (6 nM) or less. The required time for the assay will be less than 5 minutes (see Prophetic Example 1).

25 In addition to detection *B. anthracis* proteins in various samples types, a still further aspect of the invention is the detection of specific antibody to PA, LF or EF in bodily fluids by an assay method incorporation FP, FLT or FRET technology. The bodily fluids

5 that are included as an aspect of the invention include: saliva, oral rinse expectorant, oral fluid including oral mucosal transudate and gingival crevicular fluid, urine, sweat, tears, blood, serum, stool, gastric fluid, synovial fluid, phlegm, and other clinical and laboratory specimens and samples.

The inventive subject matter for the detection of specific antibodies to *B. anthracis*
10 proteins includes the steps:

- a. intermixing a sample, suspected of containing specific antibody with a competitive reagent labeled with a fluorochrome, capable of binding to the specific antibody;
- b. incubating the sample, containing specific antibody, and competitive
15 reagent for 15 seconds to 5 minutes;
- c. detecting binding interaction between said protein and antibody.

Detection of specific agent would then be by either a change in fluorescence polarization or a change in fluorescence life-time, depending on whether the technology incorporated was FP or FLT, respectively. If the method incorporated FRET, then
20 detection is by sensitized fluorescence of the acceptor or by quenching of donor fluorescence or by fluorescence depolarization.

The results of test samples will be compared to results obtained from negative or positive control results, using either FP, FLT or FRET technology. Negative controls will contain the same fluorochrome-labeled competitive reagent as used in the test
25 sample. A positive control can also contain the same fluorochrome-labeled competitive reagent as in the test sample but will be exposed to a known amount of said protein. The

5 measured results of the test samples will then be compared to either or both of the negative or positive controls.

It is estimated, like for detection of antigen, the assay will take less than 5 minutes to complete. Less time will be required for samples with high antibody titers (30 seconds or less incubation time). Also, like for detection of antigen, an inventive aspect is the
10 ability to select fluorochromes, depending on sample conditions, including pH-independent fluorochromes. The fluorochromes available, as an aspect of the invention, include: 7-AAD, Acridine Orange, Alexa 488, Alexa 532, Alexa 546, Alexa 568, Alexa 594, Aminonaphthalene, Benzoxadiazole, BODIPY 493/504, BODIPY 505/515, BODIPY 576/589, BODIPY FL, BODIPY TMR, BODIPY TR, Carboxytetramethylrhodamine,
15 Cascade Blue, a Coumarin, Cy2, CY3, CY5, CY9, Dansyl Chloride, DAPI, Eosin, Erythrosin, Ethidium Homodimer II, Ethidium Bromide, Fluorescamine, Fluorescein, FTC, GFP (yellow shifted mutants T203Y, T203F, S65G/S72A), Hoechst 33242, Hoechst 33258, IAEDANS, an Indopyras Dye, a Lanthanide Chelate, a Lanthanide Cryptate, Lissamine Rhodamine, Lucifer Yellow, Maleimide, MANT, MQAE, NBD,
20 Oregon Green 488, Oregon Green 514, Oregon Green 500, Phycoerythrin, a Porphyrin, Propidium Iodide, Pyrene, Pyrene Butyrate, Pyrene Maleimide, Pyridyloxazole, Rhodamine 123, Rhodamine 6G, Rhodamine Green, SPQ, Texas Red, TMRM, TOTO-1, TRITC, YOYO-1, vitamin B12, flavin-adenine dinucleotide, and nicotinamide-adenine dinucleotide.

25 For both detection of *B. anthracis* antigen as well as antibody, specific for *B. anthracis* proteins, an aspect of the invention is that a fluorochrome concentration of 1 nM or less will yield a sample millipolarization increase or decrease of at least 10 mP. A

5 further aspect is that the method will have a specificity of 96-99% for the detection of specific antibody or antigen in bodily fluids or culture.

Prophetic Example 1

Competitive FP assay for measuring broth protective antigen, lethal factor and edema factor of *Bacillus anthracis*

10 Quantitative, real-time monitoring of growing bacterial cultures is necessary to ensure maximum production of important bacterial proteins in vaccine production. *Bacillus anthracis* is of particular importance due to its potential public health and military focus as a potential bioterrorism weapon. Monitoring of growing cultures of *B. anthracis* is conducted by the following steps:

- 15 a) Add 10 µl of labeled competitor peptide (e.g. FITC labeled PA, lethal factor or edema factor) in 1 ml buffer;
- b) Read blank;
- c) Add 20 µl of monoclonal antibody to target protein in said 1 ml buffer;
- d) Incubate for 2 minutes;
- 20 e) Read the FP of the tracer;
- f) Add 10 µl of culture broth to said 1 ml buffer in step d);
- g) Read decrease in mP. The decrease in mP is proportional to the PA concentration.

Culture broths of *B. anthracis* contain protective antigen concentrations up to 5
25 ug/ml. Therefore the assayable range of protective antigen required for FP assay is similar to that for ELISA assay. Using standard sandwich ELISA techniques, recombinant PA reports approximately 0.01 OD per 1 ng PA at 405 nm using horse

5 radish peroxidase substrate conjugated antibodies in a range of 1.5-200 ng protective antigen. An example standard ELISA was used to estimate protective antigen in serial dilutions of buffer from 25 nM (2 ug/ml) down to 0.2 nM (16 ng/ml).

Similar results are expected in competitive fluorescence polarization as immunoassay techniques (see Figure 1). Combining an fluorescent dye-labeled protective antigen probe at about 1 nM (labeled PA probe) and a monoclonal (MAb) or polyclonal (PAb) antibody at an adequate dilution (usually between 1:25-1:250K) would report numbers around 200 mP (left side of equation and left side of graph as shown in Figure 1). Culture broth would be added as unlabeled competitor (broth PA) in a range of 0.01 – 0.1 ml depending on how long the culture had grown causing dissociation of the labeled probe from the antibody. Dissociation of the labeled probe from the antibody in the presence of increasing amounts of broth PA results in increasing amounts of free labeled probe and decreasing mP readings that are proportional to the concentration of protective antigen in the culture broth. (Right side of the equation and right side of the graph). At full growth, the assay mixture would require 0.01 ml broth (5 ug/ml protective antigen, about 6 nM) while partial growth would require proportionately more broth up to 0.1 ml to estimate a similar concentration. Other optional reagents may be used to suppress intrinsic culture broth fluorescence.

Example 2

25 Determining the concentration of recombinant or native protective antigen (PA) of

Bacillus anthracis antigen in fermentation broth

5 Overlapping 15 and 25 mer peptide sequences were synthesized from the amino
acid region 1-764 of full-length PA using Fmoc (9-fluorenylmethoxycarbonyl) chemistry
including a final step to attach an N-terminal 6-carboxyfluorescein (FAM). Enzymatic
digests of fluorescein-6-isothiocyanate (FITC isomer II) labeled full length PA were
made with trypsin, pepsin and chymotrypsin. These peptides were screened for specific
10 antibody binding by immune sera. Using Western Blot, ELISA and dot-blot analysis, a
10-mer portion of domain 4 of the COOH region of PA was suitable as an FP probe, a 5-
6 kDa portion of domain 3 and domain 4 was suitable as an FP probe, a 10 kDa portion of
domain 3 and 4 was suitable as a FP probe, a 5-6 kDa portion of domain 1 and 2 was
suitable as a FP probe, and a 10 kDa portion of domain 1 and 2 was suitable as a FP
15 probe

Fermentation broth cultures of *Bacillus anthracis* (vaccine strain V770-NP1-R)
containing PA were produced. Culture media is then mixed with antibody and
competitive probe. Incubation times was less than 30 seconds for cultures nearing the
stationary phase of growth and for less than 4 minutes for newly seeded cultures (and
20 therefore low concentration of PA). At 1nM probe concentration, the blank subtracted
fluorescence polarization reading is determined to be quantifiable if mP decreased by at
least 10 mP.

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5 Having described the invention, one of skill in the art will appreciate in the
appended claims that many modifications and variations of the present invention are
possible in light of the above teachings. It is therefore, to be understood that, within
the scope of the appended claims, the invention may be practiced otherwise than as
specifically described.

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